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Cloning and Characterization of *GRB14*, a Novel Member of the *GRB7* Gene Family*

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Roger J. Daly‡, Georgina M. Sanderson, Peter W. Janes, and Robert L. Sutherland

From the Cancer Biology Division, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, New South Wales 2010, Australia

Screening of a human breast epithelial cell cDNA library with the tyrosine-phosphorylated C terminus of the epidermal growth factor receptor identified a novel member of the *GRB7* gene family, designated *GRB14*. In addition to a pleckstrin homology domain-containing central region homologous to the *Caenorhabditis elegans* protein F10E9.6/mig 10 and a C-terminal Src homology 2 (SH2) domain, a conserved N-terminal motif, P(S/A)IPNPFPEL, can now be included as a hallmark of this family. *GRB14* mRNA was expressed at high levels in the liver, kidney, pancreas, testis, ovary, heart, and skeletal muscle. Anti-Grb14 antibodies recognized a protein of approximately 58 kDa in a restricted range of human cell lines. Among those of breast cancer origin, *GRB14* expression strongly correlated with estrogen receptor positivity, and differential expression was also observed among human prostate cancer cell lines. A GST-Grb14 SH2 domain fusion protein exhibited strong binding to activated platelet-derived growth factor (PDGF) receptors (PDGFRs) *in vitro*, but association between Grb14 and β-PDGFRs could not be detected *in vivo*. In serum-starved cells, Grb14 was phosphorylated on serine residues, which increased with PDGF, but not EGF, treatment. Grb14 is therefore a target for a PDGF-regulated serine kinase, an interaction that does not require PDGFR-Grb14 association.

Many intracellular targets for receptor tyrosine kinases (RTKs)[1] contain one or more SH2 domains. These are conserved, noncatalytic domains of approximately 100 amino acids that bind to short peptide sequences containing phosphotyrosine (1). Since receptor autophosphorylation on specific tyrosine residues follows RTK activation, SH2 domains mediate receptor-substrate interactions as well as other protein-protein interactions during signal transduction. Since the specificity of SH2 domain binding is largely determined by amino acid residues C-terminal to the phosphotyrosine, the particular autophosphorylation sites present on a given RTK define the SH2 domain-containing signaling proteins that it can recruit and hence, to a large extent, the signaling specificity of the receptor. The CORT technique, in which cDNA expression libraries are screened with the tyrosine-phosphorylated C terminus of the EGFR, represents a powerful methodology for the identification and characterization of novel, SH2 domain-containing, receptor substrates (2–5).

SH2 domains are often accompanied in signaling proteins by two other conserved protein modules: SH3 domains, which bind to proline-rich peptide ligands with a P*XX*P core sequence (6) and thereby also mediate protein-protein interactions, and PH domains. The latter are conserved protein modules now identified in about 60 intracellular proteins, most of which either perform a signaling function or are associated with the membrane cytoskeleton (7). Despite the frequent occurrence of the PH domain and the recent definition of its three-dimensional structure (8–11) the precise role of this module remains obscure. Although several PH domains bind to the βγ subunits of heterotrimeric G proteins (12), this interaction appears to involve only the C-terminal region of the domain. Two other groups have reported protein-protein interactions mediated by PH domains; Yao *et al.* observed binding of the Btk PH domain to protein kinase C (13), whilst the PH domain of Akt contributes to homotypic oligomerization and kinase regulation (14). However, another possibility is that not all PH domain ligands are protein in nature, since several of these domains bind phosphatidylinositol 4,5-bisphosphate (15), and inositol 1,4,5-trisphosphate represents a high affinity ligand for the phospholipase C-δ_1 PH domain (16).

SH2 domain-containing proteins can be divided into two classes (17): class I, which also possess a catalytic function, *e.g.* phospholipase C-γ1 and Ras-GAP, and class II, which contain only noncatalytic protein modules and are thought to function as adaptors, linking separate catalytic subunits to receptors or other signaling proteins. A member of the latter class is Grb2, which consists of a SH2 domain flanked by two SH3 domains. The SH2 domain acts as a binding site for specific tyrosine-phosphorylated proteins including the EGFR and Shc, while the SH3 domains bind proline-rich sequences in the Ras GDP-GTP exchanger Son of Sevenless (18).

Members of the ErbB family of RTKs and their ligands are implicated both in normal mammary gland development and the growth and progression of human breast cancer (19). Furthermore, marked alterations in the expression or activity of several SH2 domain-containing proteins have been observed in human breast cancers or breast cancer-derived cell lines, suggesting that this represents an additional level at which RTK signaling may be modulated in this disease (20). We therefore chose the human mammary epithelial cell line HMEC 184 as a model system on which to base a CORT screening program and

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L76687.

‡ To whom correspondence should be addressed. Tel.: 61-2-2958100; Fax: 61-2-2958321; E-mail: r.daly@garvan.unsw.edu.au.

[1] The abbreviations used are: RTK, receptor tyrosine kinase; bp, base pair(s); CMV, cytomegalovirus; CORT, cloning of receptor targets; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; EGFR, EGF receptor; ER, estrogen receptor; FCS, fetal calf serum; Grb and *GRB*, growth factor receptor-bound; GST, glutathione *S*-transferase; kb, kilobase(s); PDGF, platelet-derived growth factor; PDGFR, PDGF receptor; PH, pleckstrin homology; PVDF, polyvinylidene difluoride; Ras-GAP, Ras GTPase-activating protein; PAGE, polyacrylamide gel electrophoresis; SH, Src homology.

hence identify novel, relatively tissue-specific, ErbB receptor targets.

MATERIALS AND METHODS

cDNA Library Construction—Total cellular RNA was prepared by the guanidinium isothiocyanate-cesium chloride procedure (21) and poly(A)$^+$ RNA isolated using Dynabeads (Dynal, Carlton South, Victoria, Australia). Double-stranded cDNA was synthesized using the ZAP-cDNA synthesis kit (Stratagene, La Jolla, CA) with the following modifications. First strand cDNA synthesis was primed using an oligo(dT)-based primer (*Hin*dIII primer-adaptor, Novagen, Madison, WI). Following second strand synthesis, *Eco*RI adaptors were ligated to the cDNA, which was then cloned into *Hin*dIII-*Eco*RI-digested λEXlox (Novagen). Two cDNA libraries were constructed. In the first library, 5-methyl dCTP was incorporated into the first strand cDNA, and the primary library (approximately 4×10^6 recombinants) was amplified on ER1647 cells prior to expression screening on strain BL21(DE3)pLysE. A second small library was also made in which the first strand cDNA was not methylated; this library (approximately 2×10^5 recombinants) was plated directly on BL21(DE3)pLysE cells and screened unamplified.

Library Screening—Approximately 1×10^6 recombinants from the amplified library and the entire unamplified library were screened by the CORT technique as described previously (5), except that duplicate plaque lifts were performed. Following the isolation of the initial *GRB14* cDNA, further clones were isolated from either the 184 cDNA library or a human λgt11 liver 5′ stretch cDNA library (Clontech, Palo Alto, CA) by standard DNA hybridization techniques (21). DNA probes were labeled by random primer extension (Promega, Sydney, NSW, Australia) using [α-^{32}P]dCTP (110 TBq/mmol, Amersham Australia Pty Ltd, Castle Hill, NSW, Australia).

Clone Characterization and DNA Sequencing—Following plaque purification of λEXlox recombinants, the corresponding pEXlox plasmid was excised using bacterial strain BM25.8. DNA minipreps were then performed, and the plasmids were transformed into DH5α for further analysis. In the case of λgt11 recombinants, the corresponding *Eco*RI inserts were subcloned into pBluescript SK+ (Stratagene) for sequencing. DNA sequencing was performed by the dideoxy chain termination method using Sequenase 2.0 (Bresatec, Adelaide, SA, Australia).

Amino acid sequence alignments were performed using the computer programs Clustal W and SeqVu.

Northern Blot Analysis—Human multiple tissue Northern blots (Clontech) were hybridized under conditions recommended by the manufacturer. For analysis of *GRB14* gene expression in cell lines, total cellular RNA was isolated, fractionated, and subjected to Northern analysis as described previously (22). Blots were hybridized with an 882-bp *Eco*RI fragment from clone 1–1 (Fig. 2*A*) labeled by random primer extension (Promega) using [α-^{32}P]dCTP (Amersham).

cDNA Expression Vectors—CMV promoter-based expression vectors for the human EGFR and the β-PDGFR were as described previously (23).

A Flag epitope-tagged Grb14 eukaryotic expression vector was constructed as follows. The complete open reading frame of *GRB14* was first assembled in the vector pRcCMV (InVitrogen Corp., San Diego, CA) using DNA restriction fragments derived from cDNA clones L5 (in pBluescript SK+) and 1–2 (in pEXlox) (Fig. 2*A*). Briefly, L5 was digested with *Spe*I (which cuts in the vector polylinker) and *Avr*II to delete a 93-bp fragment harboring an *Apa*I site from the 5′ end of this cDNA (Fig. 2*A*). The digested vector was then religated, and a restriction fragment from between the *Xba*I site in the polylinker and *Apa*I site in the cDNA prepared and cloned into pRcCMV. The *GRB14* open reading frame was then completed by cloning an *Apa*I fragment encoding the C-terminal region of Grb14 from clone 1–2 into this vector. However, the resulting expression vector produced only low yields of recombinant Grb14 in a coupled transcription/translation system (Promega). Enhanced expression of Grb14 was achieved by deletion of a GC-rich region 5′ of the translation start codon. This was achieved by synthesis of a cDNA encoding the *GRB14* open reading frame by polymerase chain reaction from the *GRB14*/pRcCMV template using oligonucleotide primers containing *Hin*dIII (5′) and *Bam*HI (3′) sites for directional cloning. This DNA fragment was inserted between the *Hin*dIII and *Bgl*II sites of pRcCMV$_{Flag}$, a modified version of pRcCMV designed for tagging of expressed proteins with the 8-amino acid Flag epitope (DYKDDDDK) (24) at the C terminus. The construction of pRcCMV$_{Flag}$ will be described in detail elsewhere. The sequence of the *GRB14* cDNA in this vector was confirmed by DNA sequencing.

Generation of GST Fusion Proteins—A DNA fragment corresponding to the SH2 domain of Grb14 (amino acids 426–540) was synthesized by polymerase chain reaction using *Grb14* cDNA as a template and flanking primers containing restriction sites suitable for in-frame insertion into pGEX2T (Amrad Pharmacia Biotech, Melbourne, Victoria, Australia). A similar strategy was used to clone the mouse Grb7 SH2 domain (amino acids 418–535) into pGEX2T, except that the cDNA encoding this region was initially synthesized by reverse transcription-polymerase chain reaction from mouse liver RNA. Following construction, the recombinant plasmids were used to transform *Escherichia coli* DH5α to ampicillin resistance. Both constructs were verified by DNA sequencing. Fusion proteins were purified from isopropyl-β-D-thiogalactopyranoside-induced bacterial cultures as described previously (25).

Cell Culture—HMEC 184 normal human mammary epithelial cells were obtained from Dr. Martha Stampfer (University of California, Berkeley). HMEC-1001–7 and HMEC-219–4 cells were obtained from Clonetics Corp. (San Diego, CA). All HMEC lines were maintained in mammary epithelial growth medium (Clonetics). Maintenance of human breast cancer cell lines was as described previously (26). The human prostate cancer cell lines DU145, LnCaP, and PC3 were obtained from the ATCC (Rockville, MD), while the human epidermoid carcinoma cell line A431 was obtained from the Ludwig Institute for Cancer Research (Melbourne, Australia). These cell lines were maintained in the same medium as the breast cancer cell lines. HEK 293 cells were obtained from the ATCC and maintained in modified Eagle's medium (CSL Biosciences, Parkville, Victoria, Australia) supplemented with Hanks' buffered salt solution, 2 mM glutamine, 6.7 mM sodium bicarbonate, and 10% FCS (CSL Biosciences). Cell lines were starved overnight in medium containing 0.5% FCS prior to preparation of cell lysates for protein analysis. HER14 and HER1–2 cells, transfected NIH3T3 fibroblasts expressing the human EGFR and EGFR/ErbB2 chimera, respectively, were maintained as described previously (27, 28). Prior to growth factor treatment the cells were starved overnight in Dulbecco's modified Eagle's medium (CSL Biosciences) containing 0.5% calf serum (CSL Biosciences).

Growth factors were used at the following final concentrations: human recombinant EGF, (Life Technologies, Inc., Glen Waverley, Victoria, Australia), 275 ng/ml; human recombinant PDGF BB (Life Technologies, Inc.), 50 ng/ml. HER14 and HER1–2 cells were stimulated with growth factors for 2 min at 37 °C, and HEK 293 cells were stimulated for 5 min at 37 °C.

Antibodies—Commercially available antibodies used were as follows: monoclonal anti-phosphotyrosine antibody PY20 (Transduction Laboratories, Lexington, KY); anti-EGFR monoclonal antibody clone Z025 (Zymed Laboratories Inc., South San Francisco, CA); anti-ErbB2 monoclonal antibody (Novocastra, Newcastle Upon Tyne, UK); anti-PDGF Type A/Type B receptor polyclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY); anti-Flag M2 monoclonal antibody (IBI, Eastman Kodak Company, New Haven, CT).

The GST-Grb14 SH2 domain fusion protein was used to raise an anti-Grb14 polyclonal antiserum in rabbits. The resulting antiserum (number 264) was affinity-purified by an adaptation of the method of Smith and Fisher (29) using a fusion protein consisting of the Grb14 SH2 domain fused to the T7 gene *10* product as an affinity reagent. This methodology provided the additional advantage of removing anti-GST antibodies in the same step. Briefly, the pEXlox plasmid containing *GRB14* clone 1 (Fig. 2*A*) was excised and transformed into *E. coli* BL21 DE3 pLysE. Lysates from isopropyl-β-D-thiogalactopyranoside-induced bacteria were separated by SDS-PAGE and transferred to nitrocellulose, and the position of the induced fusion protein was identified by Ponceau S staining. The filters were then incubated with crude antiserum diluted 1:1 in Tris-buffered saline, and following washing, the anti-Grb14 antibodies were eluted from the appropriate region of the filter by incubation in 0.1 M glycine, pH 2.5, for 10 min. The eluate was neutralized with 0.2 × volume of 1 M Tris-HCl, pH 8.0, concentrated, and finally subjected to buffer exchange with Tris-buffered saline (10 mM Tris-HCl, 150 mM NaCl, pH 7.4) using a Centricon 30 microconcentrator (Amicon, Beverly, MA). The affinity-purified antibody was then quantitated, adjusted to 0.1% bovine serum albumin, and stored at −70 °C.

Cell Lysis, Immunoprecipitation, and Western Blotting—These techniques were as described previously (30), except that a modified radioimmunoprecipitation buffer was used to wash immunoprecipitates from metabolically radiolabeled cells (3).

Densitometric analysis of autoradiographs was performed using the IP Lab Gel analysis program (Signal Analytics Corp., Vienna, Virginia).

Binding Assays—Following cell lysis, 300 μl of lysate was incubated with 5 μg of GST fusion protein coupled to glutathione-agarose beads for 2 h at 4 °C. The beads were then washed 3 × with cell lysis buffer

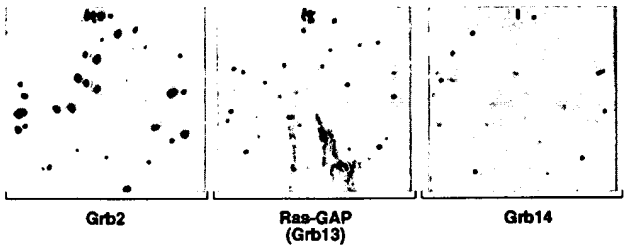

FIG. 1. **Isolation of cDNA clones encoding EGFR binding proteins by the CORT method.** A λEXlox cDNA expression library prepared from the 184 normal human mammary epithelial cell line was screened with the ^{32}P-labeled carboxyl terminus of the EGFR. The *figure* shows tertiary screening results for cDNA clones corresponding to Grb2, Ras-GAP/Grb13, and Grb14. The exposure time for the autoradiographs was 4.5 h at −70 °C with one intensifying screen.

and boiled for 3 min in SDS-PAGE sample buffer. Following separation by SDS-PAGE, bound proteins were transferred to nitrocellulose and Western blotted with anti-receptor antibodies.

Direct binding of GST fusion proteins to growth factor receptors immobilized on nitrocellulose (Far Western blotting) was performed as described previously (30), except that an anti-GST monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) was used for detection of bound fusion protein and antibody visualization was by ECL.

Transfection Procedures—Transient transfection of HEK 293 cells was performed by a modification of the calcium phosphate precipitation method (31). Briefly, cells were plated at a density of 2×10^5 cells/well of a 6-well tissue culture plate. Approximately 24 h later, DNA precipitates were added (4 μg/well), and the cells were maintained for 16 h at 3% CO_2. The medium was then replaced with 0.5% FCS/modified Eagle's medium with Hanks' buffered salt solution, and the cells were returned to 5% CO_2 prior to stimulation and/or harvesting approximately 24 h later.

Stable transfection of HEK 293 cells was performed by the same protocol scaled up to 10-cm tissue culture dishes. Following transfection the cells were subcultured 1:10 and subjected to selection with Geneticin (1 mg/ml, Life Technologies, Inc.). Individual colonies were then isolated by trypsinization using cloning cylinders.

Metabolic Labeling of Cells—Cells were plated at a density of 1.6×10^6/dish in 10-cm tissue culture dishes and 24 h later starved overnight in medium containing 0.5% FCS. Prior to metabolic labeling the cells were maintained in phosphate-free modified Eagle's medium with Earle's Salts (Life Technologies, Inc.) supplemented with 26 mM sodium bicarbonate and 0.5% dialyzed FCS (Life Technologies, Inc.) for 2 h. The cells were then washed twice in phosphate-free medium and labeled for 1.5 h in the same medium containing 20 mM HEPES, pH 7.5 and 0.5 mCi/ml ^{32}P-orthophosphate (370 MBq/ml, Amersham). Metabolic labeling following transient transfection was performed by the same methodology after the serum deprivation step.

Phosphoamino Acid Analysis—Following washing, Grb14 immunoprecipitates from [^{32}P]orthophosphate-labeled cells were separated by SDS-PAGE (8% gels), transferred to PVDF membranes (Applied Biosystems, Foster City, CA) and subjected to autoradiography. Acid hydrolysis of PVDF-bound Grb14 was then performed according to the method of Kamps (32), and phosphoamino acids were separated by one-dimensional thin layer electrophoresis at pH 3.5 on cellulose sheets (Merck, Darmstadt, Germany).

RESULTS

Screening of a Normal Breast Epithelial Cell cDNA Library by the CORT Technique—CORT screening of the two cDNA libraries prepared from strain 184 normal breast epithelial cells led to the isolation of several recombinants that exhibited differential binding to the phosphorylated EGFR C terminus; the results for some of these clones are shown in Fig. 1. Upon excision of the corresponding pEXlox plasmids and sequencing of the DNA inserts, two recombinants that bound very strongly were identified as *GRB2* cDNA clones (3), and a clone exhibiting moderate binding and designated *GRB13* according to CORT nomenclature corresponded to Ras-GAP (33). The final clone shown in the figure, *GRB14*, bound only weakly to the EGFR. A data base search with the corresponding cDNA sequence did not detect an exact match but revealed significant

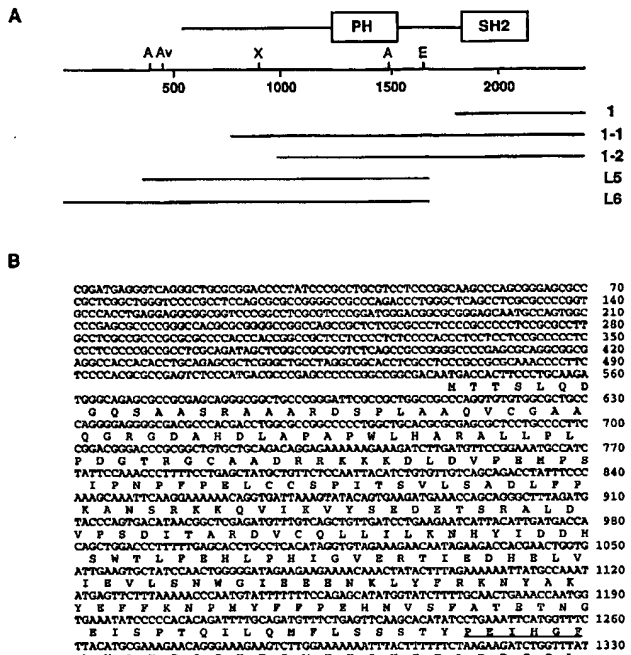

FIG. 2. **Determination of the *GRB14* cDNA sequence.** *A*, a schematic representation of *GRB14* structure with a restriction map for the *GRB14* cDNA and the cDNA clones used to derive the *GRB14* sequence aligned *below*. The initial clone isolated by CORT screening was designated clone 1. Two other clones (1-1 and 1-2) were isolated from the 184 cell line library by screening using clone 1 as a probe. The *GRB14* cDNA sequence was completed using two clones, L5 and L6, isolated from a human liver cDNA library. Abbreviations are as follows. *A*, *Apa*I, *Av*, *Avr*II, *X*, *Xho*I, *E*, *Eco*RI. The *numbers* refer to distance in bp. *B*, nucleotide and amino acid sequence of *GRB14*. The PH domain is *underlined*, and the SH2 domain is indicated by *boldface type*. The translation termination codon is shown by an *asterisk* in the amino acid sequence. *Numbers* refer to distances in bp.

sequence homology with the SH2 domain-containing protein Grb7 (2). The cDNA (*GRB14* clone 1 in Fig. 2*A*) encoded a short stretch of amino acids followed by a C-terminal SH2 domain; homology to *GRB7* was apparent over this entire open reading frame.

Characterization of Grb14—In order to obtain the full-length cDNA sequence for *GRB14*, two cDNA library screens were performed. In the first, the cDNA insert from clone 1 was used to screen the strain 184 cDNA library. Screening of 5×10^5 recombinants isolated 2 cDNAs, designated 1-1 and 1-2, of 1.6 and 1.4 kb, respectively (Fig. 2*A*). In the second, a 213-bp *Eco*RI-*Xho*I restriction fragment derived from 1-1 (Fig. 2*A*) was used to screen a human liver cDNA library. Screening of 1×10^6 recombinants isolated two cDNAs, designated L5 and L6, of 1.3 and 1.7 kb, respectively (Fig. 2*A*). Clones 1-1, 1-2, L5, and L6 were sequenced in their entirety on both strands to obtain the cDNA sequence shown in Fig. 2*B*. The 2.4 kb of DNA

FIG. 3. **Sequence homology among Grb14, Grb7, Grb10, and F10E9.6.** An alignment of the amino acid sequences of Grb14, mouse Grb7, mouse Grb10, and *C. elegans* F10E9.6 was obtained using the computer programs Clustal W and SeqVu. Identical residues are *boxed*. A highly conserved proline-rich motif is indicated by the *dotted underline*, the PH domain by the *broken underline*, and the SH2 domain by the *thick underline*. Only the central region of F10E9.6 exhibiting homology with the Grb7 family is shown. Amino acid residues for each protein are numbered (from the initiation methionine) on the *right*.

sequence derived from these overlapping clones corresponds closely to the size of the most abundant mRNA species detected upon Northern blot analysis (Fig. 4).

Analysis of the cDNA sequence identified an open reading frame of 540 amino acids. The initiation codon is preceded by an in-frame termination codon and is surrounded by a consensus sequence for strong translational initiation (34). The encoded protein is similar both in molecular architecture and amino acid sequence to Grb7 (2) and the recently identified Grb10 (4), consisting of an N-terminal region containing at least one proline-rich motif, a central region of approximately 320 amino acids, which exhibits significant homology to the *C. elegans* protein F10E9.6/mig 10 (4, 35) and which also encompasses a PH domain, and a C-terminal SH2 domain. An alignment of the amino acid sequences of Grb14, Grb7, Grb10 and F10E9.6 is shown in Fig. 3.

Grb14 is similar in size to Grb7, Grb10 possessing a more extended N terminus. The N-terminal regions of Grb14, Grb7, and Grb10 exhibit low sequence homology apart from a highly conserved amino acid motif P(S/A)IPNPFPEL. Also of note is the presence of two clusters of basic residues, which flank this motif. Overall, the N-terminal region of Grb14 displays a lower proline content than that of Grb7 and Grb10 (Grb14 amino acids 1–110, 11% proline; Grb10 amino acids 1–113, 15%; Grb7 amino acids 1–103, 23%).

In the conserved central region, Grb14 bears 48, 55, and 28% amino acid identity, respectively, with Grb7, Grb10, and F10E9.6 (Fig. 3). The core of this region is provided by a PH

TABLE I
Expression of GRB14 mRNA in different human cell lines

Total cellular RNA was extracted from the indicated cell lines and subjected to Northern blot analysis using a *GRB14* cDNA probe labeled with ^{32}P by random primer extension. The relative expression levels of *GRB14* mRNA were then scored on a scale from + (low) to ++++ (high). −, undetectable expression.

Origin	Cell line	Expression
Normal human breast epithelial	HMEC 184	+++
	HMEC-219-4	+
	HMEC-1001-7	−
Human breast cancer, ER+	T-47D	+++
	ZR-75-1	++
	MCF-7	+
	BT-483	+
	MDA-MB-134	+
	MDA-MB-361	+
	BT-474	−
Human breast cancer, ER−	MDA-MB-330	+
	MDA-MB-468	+
	BT-20	−
	SK-BR-3	−
	BT-549	−
	Hs578T	−
	DU-4475	−
	MDA-MB-157	−
	MDA-MB-175	−
	MDA-MB-231	−
	MDA-MB-436	−
	MDA-MB-453	−
Human prostate cancer	PC3	+
	LnCaP	+
	DU-145	++++
Human epidermoid carcinoma	A431	−
Human embryonic kidney	HEK 293	++++

domain (Figs. 2 and 3) (7), over which Grb14 exhibits 56, 61, and 35% amino acid identity, respectively, with Grb7, Grb10, and F10E9.6. However, as noted by Ooi *et al.* (4), another region of marked homology spanning approximately 100 amino acids exists amino-terminal to the PH domain (Fig. 3).

The most highly conserved region among Grb7 family members is the SH2 domain (Fig. 3). The Grb14 SH2 domain displays 67 and 74% amino acid identity, respectively, with the corresponding domain in Grb7 and Grb10.

Northern Blot Analysis of Grb14 Gene Expression—The tissue specificity of Grb14 gene expression was investigated by hybridizing Northern blots of poly(A)$^+$ RNA isolated from a variety of human tissues to a *GRB14*-specific cDNA probe. *GRB14* gene expression was highest in the testis, ovary, heart, liver, skeletal muscle, kidney, and pancreas. Moderate expression was detected in the small intestine, colon, peripheral blood leukocytes, brain, and placenta, while expression in the spleen, thymus, prostate, and lung was low or undetectable. Several mRNA transcripts were detected that displayed tissue-specific variation in their relative abundance. The three most prominent transcripts were approximately 2.3, 2.4, and 2.5 kb. Often co-expressed with one or two of these transcripts was a transcript of approximately 9.5 kb. In the ovary a still larger transcript of undetermined size was also expressed.

Since the *GRB14* cDNA was originally isolated from a cDNA library prepared from normal human breast epithelial cells, we were interested in determining the expression profile of *GRB14* mRNA in a panel of human breast cancer cell lines. Upon Northern blot analysis of total RNA isolated from 3 normal human breast epithelial cell strains and 19 human breast cancer cell lines, *GRB14* gene expression could be detected in HMEC 184 and HMEC-219-4 cells, 6/7 ER+ human breast cancer cell lines, and 2/12 ER− cell lines (Table I). Thus *GRB14* gene expression appears largely restricted to normal breast epithelial and ER+ breast cancer cells. Differential expression of *GRB14* was also observed among human prostate cancer cell

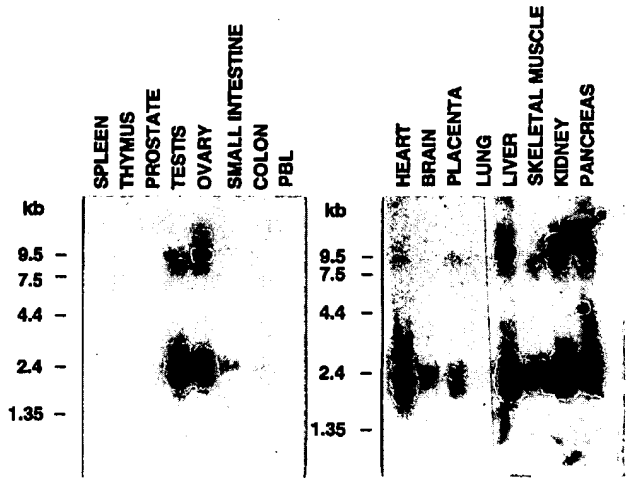

FIG. 4. **Northern blot analysis of Grb14 gene expression.** Northern blots of poly(A)$^+$ RNA isolated from a variety of human tissues were hybridized to a *GRB14* cDNA probe labeled with ^{32}P by random primer extension. The exposure time for the autoradiographs was 7 days at −70 °C with two intensifying screens.

lines. Although *GRB14* mRNA expression was undetectable in the normal prostate (Fig. 4), low expression could be detected in the PC3 and LnCaP prostate cancer cell lines and high expression in the DU145 line (Table I).

Expression of Grb14 Protein—In order to characterize the Grb14 protein a polyclonal antiserum was raised against a GST-Grb14 SH2 domain fusion protein. Following affinity purification, this antiserum was used to Western blot cell lysates derived from cell lines in which *GRB14* mRNA was either expressed at high levels (DU145 and HEK 293) or was undetectable (A431 and SK-BR-3) (Table I). This antiserum recognized a protein of approximately 58 kDa in DU145 cells, while in HEK 293 cells a tight doublet of this mobility was detected (Fig. 5*A*). These bands were not observed upon Western blotting with preimmune serum or in the cell lines that do not express *GRB14* mRNA. This estimated size of Grb14 upon SDS-PAGE is in accordance with the predicted size of the translation product of the *GRB14* cDNA (60 kDa). Although Grb14 is of similar size to Grb7, the Grb14 antiserum does not significantly cross-react with this protein, which is expressed at high levels in SK-BR-3 cells (35).

Since DU145 cells overexpress *GRB14* mRNA relative to the two other prostate carcinoma cell lines examined (Table I), we investigated whether this was accompanied by an up-regulation of Grb14 protein expression. Upon Western blot analysis, Grb14 was clearly detectable in DU145, but not PC3 or LnCaP, cell lysates (Fig. 5*B*), indicating that Grb14 protein is overexpressed in this cell line.

Interaction of Grb14 with Activated Growth Factor Receptors—It appears likely that the Grb7 family function as adaptors, binding activated receptors or other tyrosine-phosphorylated proteins via their SH2 domains and recruiting effector proteins via other interaction domains. It was therefore of interest to determine the specificity of Grb14 SH2 domain binding and to compare it with the SH2 domain of another family member, Grb7.

When compared with either the Grb7 (Fig. 6) or the Grb2[2] SH2 domains for binding to the EGFR, the Grb14 SH2 domain exhibited a relatively weak interaction, as might be predicted from the CORT screening results (Fig. 1). In accordance with other RTK-SH2 domain interactions, the binding of the Grb14 SH2 domain was dependent on ligand stimulation and tyrosine phosphorylation of the receptor. A difference in binding selec-

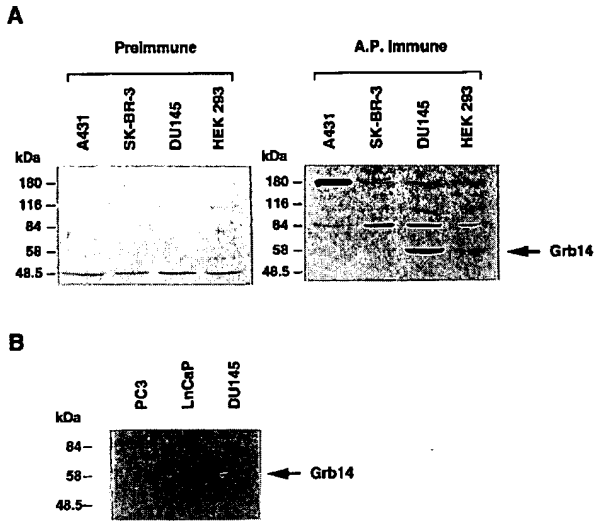

FIG. 5. **Expression of Grb14 protein in different human cell lines.** *A*, detection of Grb14 by Western blot analysis. Lysates were prepared from the indicated cell lines and equivalent amounts of protein separated by SDS-PAGE (10% gels), transferred to nitrocellulose, and Western blotted with either preimmune serum or affinity-purified (*A.P.*) antiserum 264. Detection of bound antibody was by ECL. The mobility of molecular weight standards was as indicated. *B*, expression of Grb14 in human prostate cancer cell lines. Lysates were prepared from the indicated cell lines and equivalent amounts of protein subjected to Western blot analysis with affinity purified antiserum 264 as described previously.

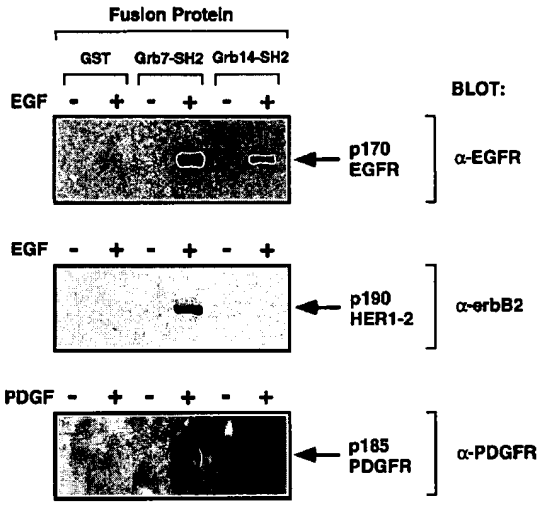

FIG. 6. **Interaction of the Grb14 SH2 domain with different RTKs.** *Top panel*, comparison of Grb14 SH2 domain binding to the EGFR with that of Grb7. Lysates from either control (−) or EGF-stimulated (+) HER14 cells were incubated with GST, GST-Grb7 SH2, or GST-Grb14 SH2 coupled to glutathione-agarose beads. Following washing, bound proteins were subjected to SDS-PAGE (8% gel), transferred to nitrocellulose, and blotted with anti-EGFR antibodies. Detection of bound antibodies was by ECL. *Middle panel*, comparison of Grb14 SH2 domain binding to the HER1–2 receptor with that of the Grb7 SH2 domain. The experimental protocol was as for the *top panel* except that HER1–2 cells were used, and the detection of bound receptor was performed with an anti-ErbB2 antibody. *Lower panel*, comparison of Grb14 SH2 domain binding to activated PDGFRs with that of the Grb7 SH2 domain. The experimental protocol was as for the *top panel* except that HER 14 cells were stimulated with PDGF BB, and the detection of bound receptors was performed with anti-α/β-PDGFR antibodies.

tivity between the Grb7 and Grb14 SH2 domains was more evident when binding to the activated HER 1–2 receptor (an EGFR/ErbB2 chimera containing the intracellular domain of ErbB2) was investigated. In this experiment the Grb7 SH2 domain bound avidly to the chimeric receptor, as reported by Stein *et al.* (35), while binding of the Grb14 SH2 domain could not be detected (Fig. 6). However, both the Grb7 and Grb14 SH2 domains exhibited strong binding to activated PDGFRs (Fig. 6), which was more pronounced than that observed with the Grb2 SH2 domain.[2] In Far Western blotting experiments in which specific PDGFRs were immunoprecipitated, separated by SDS-PAGE, transferred to nitrocellulose, and then incubated with soluble GST-Grb14 SH2 domain, direct binding was observed to both activated α- and β-PDGFRs.[2]

The interaction between Grb14 and RTKs *in vivo* was studied using two systems: DU145 cells, which express high levels of endogenous Grb14 and 1.5×10^5 EGFRs/cell (36) and transient transfection of HEK 293 cells.[2] Western blotting of EGFR immunoprecipitates from EGF-stimulated DU145 cells with anti-Grb14 antiserum did not detect association between these two proteins. Furthermore, upon transient co-expression of the EGFR and Flag epitope-tagged Grb14 in HEK 293 cells, the EGFR could not be detected in Grb14 immunoprecipitates from EGF-stimulated cells. Similarly, following co-transfection of the *β-PDGFR* and *GRB14* cDNAs into HEK 293 cells, Grb14 could not be detected in anti-β-PDGFR immunoprecipitates from PDGF BB-stimulated cells, and vice versa. Therefore, true *in vivo* binding partners for the Grb14 SH2 domain remain to be identified.

Phosphorylation of Grb14—In order to characterize further the role of Grb14 in RTK signaling, the phosphorylation state of Grb14 was investigated before and after growth factor stimulation. Since the anti-Grb14 antiserum 264 did not immunoprecipitate Grb14 under either native or denaturing conditions, we utilized an expression construct ($pRcCMV_{Flag}$), which tagged Grb14 with the 8-amino acid Flag epitope at the C terminus. This construct was stably transfected into HEK 293 cells, leading to the isolation of stable clones of cells expressing an epitope-tagged Grb14, which could be immunoprecipitated with the M2 anti-Flag monoclonal antibody and Western blotted with either this antibody or anti-Grb14 antiserum 264 (Fig. 7, *A* and *B*). Immunoprecipitation of Grb14 from serum-starved cells that were metabolically labeled with ^{32}P-orthophosphate demonstrated that Grb14 was phosphorylated in this basal state (Fig. 7*C*). Phosphoamino acid analysis of the isolated protein demonstrated that phosphorylation was on serine residues (Fig. 7*D*; more prolonged exposures obtained using a PhosphorImager also only detected serine phosphorylation).

Treatment of the cells with EGF did not significantly increase this level of phosphorylation (Fig. 8*A*), although activation of native EGFRs could be demonstrated by anti-phosphotyrosine blotting of the cell lysates.[2] However, stimulation with PDGF BB resulted in an approximately 1.5-fold increase within 5 min of administration, and transient transfection of a cDNA encoding β-PDGFRs into the cells further amplified this response to approximately 2-fold (Fig. 8*B*). The small increase in phosphorylation that occurred when this construct was present in the absence of PDGF BB was presumably due to the constitutive activation of RTKs often observed with this system (23). Phosphoamino acid analysis demonstrated that the PDGF-induced increases in Grb14 phosphorylation also occurred on serine residues.[2]

DISCUSSION

This paper describes the expression cloning of a novel member of a family of SH2 domain-containing signaling proteins which contains two other proteins cloned by the CORT screening technique, Grb7 (2) and Grb10 (4). The cloning of *GRB14*

[2] R. J. Daly, G. M. Sanderson, and P. W. Janes, unpublished results.

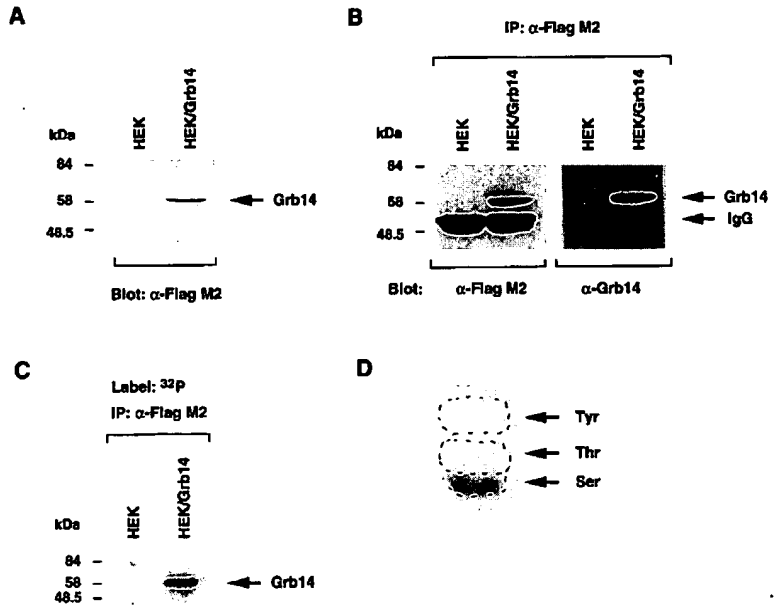

FIG. 7. **Characterization of Flag epitope-tagged Grb14 expressed in HEK 293 cells.** *A*, detection of epitope-tagged Grb14 by Western blot analysis. Lysates were prepared from control untransfected (HEK) and *GRB14*/pRcCMV$_F$ transfected (HEK/Grb14) HEK 293 cells. Equivalent amounts of protein were then separated by SDS-PAGE (10% gel), transferred to nitrocellulose, and Western blotted with anti-Flag monoclonal antibody M2. Detection of bound antibody was by ECL. *B*, immunoprecipitation of epitope-tagged Grb14. Lysates were prepared from control and transfected HEK 293 cells. Equivalent amounts of protein were then immunoprecipitated (*IP*) with anti-Flag antibody M2 and subjected to SDS-PAGE (10% gel). Following transfer to nitrocellulose the immunoprecipitates were Western blotted with affinity-purified anti-Grb14 antiserum 264 or anti-Flag antibody M2. *C*, detection of Grb14 phosphorylation. Control and transfected HEK 293 cells were serum-starved and then metabolically labeled with ^{32}P-orthophosphate. Following immunoprecipitation with anti-Flag antibody M2 and SDS-PAGE, the immunoprecipitates were transferred to a PVDF membrane and subjected to autoradiography. The exposure time was 16 h at −70 °C with two intensifying screens. *D*, detection of Grb14 serine phosphorylation. Following detection of PVDF-immobilized phosphorylated Grb14 the band was excised and subjected to phosphoamino acid analysis as described under "Materials and Methods." The mobilities of the phosphoamino acid standards phosphotyrosine (*Tyr*), phosphothreonine (*Thr*), and phosphoserine (*Ser*) were as indicated. The exposure time was 14 days at −70 °C with two intensifying screens. An equivalent of a more prolonged exposure achieved using a PhosphorImager also only detected serine phosphorylation.

provides further evidence for the strength of the CORT screening strategy in the identification of novel EGFR binding proteins. In addition to the three members of the *GRB7* gene family characterized so far, utilization of this technique also led to the cloning of *GRB1/p85* (5) and *GRB2* (3). Other SH2 domain-containing proteins isolated using this procedure include phospholipase C-γ1, Fyn, Nck-like and Crk-like proteins (2), Syp (37), and Ras-GAP (this manuscript). Furthermore, a novel, non-SH2 Tyr(P) interaction (PI) domain in Shc was also initially identified by this technique (38). Interestingly, CORT screening of the 184 cell line cDNA library also led to the isolation of four cDNAs corresponding to a protein lacking clearly recognizable SH2 or PI motifs.[2]

The Grb7 family belong to the adapter subclass of SH2 domain-containing proteins and contain at least three "interactive" protein domains likely to participate in this function. First, the cloning of *GRB14* has highlighted an N-terminal proline-rich motif P(S/A)IPNPFPEL, which is completely conserved in all three family members (Fig. 3). This is particularly striking when considered in the context of the poor sequence conservation elsewhere in the N termini of these proteins. The motif conforms to the consensus P*XX*P SH3 domain binding motif described by Yu *et al.* (6), suggesting that recruitment of proteins containing a particular subclass of SH3 domain is fundamental to Grb7 family signaling. Interestingly, Grb7 and Grb10 also contain other proline-rich regions harboring P*XX*P motifs that may provide additional SH3 domain binding sites; however, these are not conserved between the two proteins.

The second region probably involved in intermolecular interaction is the central region of approximately 320 amino acids bearing homology to the *C. elegans* protein F10E9.6. A key feature of this region is a PH domain, which may mediate protein-protein or protein-phospholipid interactions (7, 15, 39) and hence regulate signaling events and/or subcellular localization. However, since homology with F10E9.6 extends outside the PH domain (in particular to the 100-amino acid region amino-terminal to this domain) it seems likely that other sections of the central region also participate in a conserved signaling function. The only clue to the role of this region comes from the recent identification of F10E9.6 as the product of the *mig10* gene in *C. elegans* (4, 35), which is required for longitudinal neuronal migration in embryos (40). However, Grb7 family members and F10E9.6/mig 10 do not exhibit overall structural similarity, the latter containing an unrelated N-terminal region and a proline-rich C terminus flanking the central domain. It therefore remains possible that this central domain represents a protein module found in functionally distinct proteins and that the Grb7 family and F10E9.6/mig 10 perform unrelated signaling roles.

The third interactive domain identified in Grb14 is the SH2 domain. When expressed as a GST fusion protein and utilized for *in vitro* binding experiments, this domain exhibited a high affinity for a subclass of tyrosine-phosphorylated growth factor receptors, binding strongly to activated PDGF receptors in HER14 cells but only weakly to activated EGF receptors (Fig. 6). Interaction with an EGFR-ErbB2 chimera was undetectable. This represents a marked difference in binding specificity to the Grb7 SH2 domain, which binds strongly *in vitro* to the EGF and ErbB receptors (35, 38). The Grb10 SH2 domain, which is more closely related to the Grb14 SH2 domain than that of Grb7, also displays a relatively weak interaction with the EGFR (4). This gene family therefore represents an interesting model system in which to study determinants of SH2 domain binding selectivity. Indeed, we have recently identified

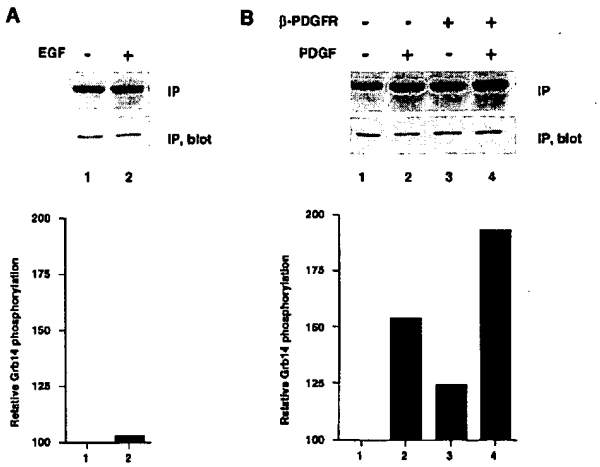

FIG. 8. **Regulation of Grb14 serine phosphorylation.** *A*, regulation by EGF. HEK 293 cells expressing Flag epitope-tagged Grb14 were serum-starved, metabolically labeled with ^{32}P-orthophosphate, and then either left untreated (−) or stimulated with EGF (+) for 5 min at 37 °C. Following cell lysis, equivalent amounts of protein were immunoprecipitated with anti-Flag antibody M2. Three-quarters of each immunoprecipitate were separated by SDS-PAGE (8% gel), transferred to a PVDF membrane and subjected to autoradiography (*IP*). The remainder was Western blotted with affinity-purified anti-Grb14 antiserum 264 (*IP, Blot*). Following densitometric analysis the amount of Grb14 phosphorylation was normalized for the Grb14 content of each immunoprecipitate and expressed as a percentage of the value for the untreated sample (*lower panel*). *B*, regulation by PDGF. HEK 293 cells expressing Flag epitope-tagged Grb14 were transiently transfected with either vector alone (*lanes 1* and *2*) or a β-PDGFR-encoding expression vector (*lanes 3* and *4*). Following serum starvation the cells were metabolically labeled with ^{32}P-orthophosphate and then either left untreated (−) or stimulated with PDGF BB (+) for 5 min at 37 °C. Determination of the relative amount of Grb14 phosphorylation was as described previously.

two amino acid residues in Grb14 that, when changed to their Grb7 counterparts, confer high affinity *in vitro* binding to ErbB2.[3] However, we have not detected association between either the EGFR or β-PDGFR and Grb14 *in vivo*, even upon transient co-expression in HEK 293 cells. Binding of the Grb14 SH2 domain to tyrosine-phosphorylated receptors/proteins may therefore be restricted by the subcellular localization, conformation, and/or phosphorylation of the full-length protein as well as the inherent binding selectivity of this domain. Recently, two potential *in vivo* partners for the Grb10 SH2 domain, the insulin and Ret RTKs, were identified by two-hybrid screens (41, 42), and the interaction of these proteins with Grb14 is currently under investigation.

Although *in vivo* association between the β-PDGFR and Grb14 could not be demonstrated, a role for Grb14 in signaling events initiated by this receptor class was demonstrated by the increase in Grb14 serine phosphorylation observed upon PDGF BB activation of native PDGF receptors or transfected β-PDGFRs (Fig. 8*B*). Interestingly, Grb10 also exhibits a basal level of serine phosphorylation that increases upon PDGF treatment without detectable PDGFR recruitment (4). However, the phosphorylation of Grb10, but not of Grb14, also increases in response to EGF stimulation (4). Whether this represents a difference in signaling specificity between these two family members or reflects the different cell types utilized in these experiments is unknown at present. The growth factor-induced serine phosphorylation of Grb10 was not mimicked by phorbol ester treatment (4), demonstrating that signaling via conventional or novel protein kinase C isoforms was not involved (43).

[3] P. W. Janes, R. L. Sutherland, and R. J. Daly, manuscript in preparation.

Since PDGF is more potent at increasing Grb14 phosphorylation than treatment with 12-*O*-tetradecanoylphorbol-13-acetate,[2] this strongly suggests that signaling via phorbol ester-activated protein kinase C isoforms is also not the major mechanism for PDGF-induced Grb14 phosphorylation. Determination of the sites of phosphorylation of Grb14 and their degree of conservation among the Grb7 family will provide further insight into the role of phosphorylation in regulation of the function of these proteins. Also, further characterization of the serine kinase involved and its interaction with Grb14 will also help determine whether this activity represents an integral component of the Grb14 signaling complex.

The *GRB7* gene family exhibit relatively tissue-specific patterns of expression. *GRB14* probably represents the most widely expressed family member and *GRB7* the least, expression of the latter gene being restricted to kidney, liver, and gonads (2). These proteins therefore differ from other "adapter" SH2 domain-containing proteins such as Grb2 (3) and Nck (44), which are ubiquitous in their expression. This suggests that the Grb7 family performs a relatively specialized signaling role, with the individual members functioning in a tissue-restricted manner to link specific receptors to effector molecules. This hypothesis is supported by the differences in SH2 domain specificity exhibited by the different family members. However, Grb7 family proteins also exhibit a provocative expression profile among different types of human cancer cells. For example, due to the close proximity of their respective genes and hence coordinate gene amplification, Grb7 is overexpressed along with its binding partner ErbB2 in a subset of human breast cancers (35). Furthermore, our results demonstrate a correlation between *GRB14* expression and estrogen receptor positivity in human breast cancer cells and, in a preliminary investigation of human prostate cancer cell lines, marked overexpression in DU145 cells *versus* the normal prostate and the other prostate cancer lines examined (Table I and Fig. 5). These and other data demonstrating aberrant expression of signaling proteins in human cancer cells (20) identify novel mechanisms for modulation of RTK signaling during tumorigenesis and highlight a potential role for such proteins as tumor markers and/or prognostic indicators.

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